Dietary supplementation with ferric tyrosine improves zootechnical performance and reduces caecal *Campylobacter* spp. load in poultry

D. Currie [1], M. Green[2], O. A. Dufailu[2], M. Pitoulias[2], P. Soultanas[2], E. McCartney[3], H. Lester [3], Liza Van den Eede[3], J. Apajalahti[4] and J. Mahdavi [2]

[1] *Roslin Nutrition Ltd., Gosford Estate, Aberlady, EH32 OPX, East Lothian, Scotland,*

[2] *School of Chemistry, Centre for Biomolecular Sciences, University of Nottingham, UK.*

[3] *Pen & Tec Consulting S.L.U., Pl. Ausias March 1, 4ª Planta, D01, 08195, Sant Cugat del Vallès, Barcelona, Spain,*

[4]*Alimetrics Group Ltd., Koskelontie 19B, FIN-02920, Espoo, Finland*

Corresponding authors:

Hannah Lester. E-mail: Hannah@pentec-consulting.eu

Jafar Mahdavi. E-mail: Jafar.Mahdavi@nottingham.ac.uk

Short title: Effect of ferric tyrosine in broilers

Abstract –

1. The objective of this study was to evaluate the effect of ferric tyrosine on the reduction of *Campylobacter* spp. and zootechnical performance in broilers exposed to *Campylobacter* spp. using a natural challenge model to simulate commercial conditions. Additionally, the minimum inhibitory concentrations (MIC) of ferric tyrosine against common enteropathogens were evaluated.
2. On day 0, 840 healthy male day-old birds (Ross 308) were randomly allocated to 6 replicate pens of 35 birds and fed diets containing different concentrations of ferric tyrosine (0, 0.02, 0.05 and 0.2 g/kg) in mash form for 42 days.
3. Overall, broilers fed diets containing ferric tyrosine showed significantly improved body weight at day 42 and weight gain compared to the control group. However, birds fed ferric tyrosine ate significantly more than the control birds so significant improvements in FCR were not observed.
4. Microbiological analyses of caecal samples collected on day 42 of the study showed, per gram sample, 2-3 $\log_{10}$ reduction in *Campylobacter* spp. and 1 $\log_{10}$ reduction in *Escherichia coli* in the groups fed diets containing ferric tyrosine compared to the control.

5. The MIC of ferric tyrosine was >400 mg/L for *C. jejuni* and >200 mg/L for *E. coli* and *Salmonella enterica*, indicating that ferric tyrosine does not exert antimicrobial activity.
6. Collectively, these results show that birds fed ferric tyrosine grew faster and consumed more feed compared to the control birds indicating potential benefits of faster attainment of slaughter weight with no significant reduction on feed efficiency. Moreover, ferric tyrosine significantly reduces caecal *Campylobacter* spp. and *E. coli* indicating potential as a non-antibiotic feed additive to lower the risk of *Campylobacter* infections transmitted through the food chain.

Keywords: *Broilers, Campylobacter, control, ferric tyrosine, iron chelates*

Introduction

Campylobacteriosis is the most common human food-borne illness in the European Union (EU) (EFSA, 2017a) and along with other enteropathogenic bacteria such as *Salmonella* spp. and *Escherichia coli* (Chaveerach et al., 2004b; Santini et al., 2010; Hermans et al., 2011), *Campylobacter* spp. pose a serious public health risk. Contaminated chicken meat is a major source of human infection (Freidman et al., 2004; Adak et al., 2005; Bull et al., 2008), with *ca.* 200,000 reported cases of campylobacteriosis per year (EFSA, 2016). It is estimated that 75% of EU broiler meat samples are contaminated with *Campylobacter* spp. (EFSA, 2010). *Campylobacter* prevalence can be very high in poultry flocks, and is maintained along the food chain (EFSA, 2010, 2011). Reducing the number of contaminated carcasses entering the food chain will reduce the incidence of human cases of campylobacteriosis, hence *Campylobacter* control measures must be implemented on poultry farms to reduce human exposure (EFSA, 2011). It is estimated that reducing caecal *Campylobacter* numbers by 3 $\log_{10}$ CFU/g reduces the public health risk by 90% (Romero-Barrios et al., 2013). However, controlling *Campylobacter* on farms poses several serious challenges. A single bird infected with low numbers of *Campylobacter* can infect a whole flock (Stern et al., 2001). Furthermore, chickens appear asymptomatic meaning that infection can go undetected (EC, 2017). Strict biosecurity measures have

65 proven to be effective in excluding *Campylobacter* from housed flocks in northern Europe
66 and the United Kingdom, but are difficult to maintain in the long-term under normal
67 farming conditions (ACMSF, 2004; Bull et al., 2008). Antibiotics are no longer a viable
68 option for control and are subject to global pressure to reduce use drastically, due to
69 growing concerns about antimicrobial resistance (AMR). EFSA has recently reported that
70 *Campylobacter* strains isolated from humans and pigs are resistant to ciprofloxacin and
71 tetracyclines, critically important antibiotics for human use (EFSA, 2017b). Similar data
72 were also reported for *Salmonella* spp. and *E. coli* isolates from fattening pigs,
73 highlighting the growing problem of AMR. The EU banned the use of antibiotics as
74 growth promoters in animal feeds in 2006 (EMA/EFSA, 2017) hence, there is an urgent
75 need for alternatives to antibiotics that can protect farm animals and limit the
76 establishment and growth of bacterial pathogens, in particular zoonotic micro-organisms.
77 Various feed additives have been proposed to reduce *Campylobacter* colonization in
78 chickens, including probiotics, prebiotics, organic acids, bacteriophages, bacteriocins,
79 and plant-derivatives, some of which have shown promising results (Hermans et al., 2011;
80 Guyard-Nicodème et al., 2015). Recently, in-feed chelated iron (III) complexes have
81 shown to be effective against *Campylobacter* and other pathogenic bacteria in broilers
82 (Khattak et al., 2018). However, in the study performed by Khattak et al., birds were

artificially challenged, so the purpose of the present study was to investigate whether iron chelates have comparable effects under more natural infection conditions. The aim of the present study was to evaluate the effect of ferric tyrosine (TYPLEX®, Akeso Biomedical Inc.) on broiler zootechnical performance and reduction of caecal *Campylobacter* spp. using birds naturally infected with *Campylobacter* spp. to simulate farm conditions. Additionally, the minimum inhibitory concentrations (MIC) of ferric tyrosine against common enteropathogens were evaluated to ascertain whether ferric tyrosine exerts antimicrobial activity.

Material and Methods

Experimental birds and diets

The study protocol was approved by the Roslin Nutrition Ltd. Ethical Review Committee and the UK Food Standards Agency (FSA). The study birds were managed and handled in compliance with local animal welfare standards and Directive 2010/63/EU.

A total of 1,100 pre-sexed, male day-old broilers (Ross 308) were purchased from a local commercial hatchery and delivered to the trial site (56.0092ºN, 2.8594ºW) in Aberlady, Scotland. The trial site was an experimental research facility with animal housing set up to simulate commercial conditions. Any chicks showing signs of ill-health, injury or in poor condition were excluded from the selection process. The poultry house was lit by

102 programmable artificial light. The standard lighting program was 23 hours of light per
103 day, followed by 1-hour dark. Environmental conditions during the trial (temperature,
104 humidity and ventilation rate) were automatically controlled and appropriate for the age
105 of the broilers.
106
107 Ferric tyrosine (TYPLEX®, Akeso Biomedical, Inc.) is an organo-iron complex of iron
108 (III) complexed with L-tyrosine (4-hydroxyphenylalanine). The dietary treatments are
109 summarised in Table 1. Control group (T1) was fed the basal diets (starter and grower).
110 The treated groups received the basal diets supplemented with ferric tyrosine at 0.02 g/kg
111 (T2), 0.05 g/kg (T3) or 0.20 g/kg feed (T4). Birds were fed a starter diet from 0 to 21 days
112 and a grower diet from 21 to 41 days. All diets were formulated according to
113 recommended specifications (NRC, 1996) then analysed (AOAC, 2007) for crude
114 protein, ether extract, dry matter, iron and ash (Tables 2 and 3). Coloured tracers (Micro-
115 Tracers Inc., San Francisco) were added to ferric tyrosine at 10% w/w, to enable visual
116 confirmation of ferric tyrosine content in feeds. Proximate analyses of feed samples
117 confirmed that feed nutrients were within expected ranges. Diets did not contain any other
118 added iron compounds, coccidiostats or veterinary antibiotics. Feed and water were
119 offered *ad libitum*.

Study design

On day 0, out of a pool of 1,100 birds, 840 healthy birds were randomly allocated to four treatment groups: Control (T1), ferric tyrosine at 0.02 g/kg feed (T2), 0.05 g/kg (T3) or 0.20 g/kg feed (T4) with six replicate pens per group, each pen containing 35 birds, according to a randomised complete block design. The birds were weighed by pen on arrival and then on 21 and 42 days of trial. Individual bird body weight (BW) was calculated by dividing the average weight of the pen by the number of birds. Feed consumption and feed refusals were recorded by pen on day 21 and 42. Mortality/culls were recorded daily. Average pen weight gain (AWG), feed intake (AFI) and feed conversion rate (FCR, feed/gain) were calculated for periods 0-21, 22-42 and 0-42 days on trial. At study end (42 days on trial), five birds/pen were humanely euthanized and caecal samples were collected and sent for microbiology. The trial terminated after 42 days and all birds were humanely euthanised by cervical dislocation and the carcasses destroyed.

Campylobacter spp. challenge

A natural *Campylobacter* challenge model was used whereby study birds were bedded on fresh wood shavings, over which litter from the previous batch of broilers was laid. This natural challenge model was developed at Roslin Nutrition. The natural challenge model

was selected to replicate as far as possible, a natural infection under commercial conditions. Litter samples from previous batches of birds taken from the barn used for this study had tested positive for *Campylobacter* spp. Furthermore, birds previously housed in this barn had tested positive for *Campylobacter* spp. on several occasions.

Microbiology

On day 42, five birds per pen were humanely euthanised by cervical dislocation. The caeca from each individual bird were removed and tied off to preserve caecal contents, placed in a pre-labelled zip-lock bag and immediately placed on dry ice. The birds were processed in descending order of ferric tyrosine concentration with the control birds processed last to reduce the likelihood of cross-contamination. Sterile equipment was used and changed between each treatment group. Latex gloves were worn by study staff responsible for the removal of the caeca and were changed between treatment groups. The samples were sent *via* courier to the microbiology laboratory for *Campylobacter* spp. and *E. coli* enumeration by conventional culture. Caeca were stored frozen (-80ºC) until analysis. Prior to analysis, the caecal samples were removed from the freezer and allowed to defrost. A sterile scalpel was used to cut off the blind end of both caecal sacks. From each caecal sack, 0.5 gram of caecal contents, in total 1g, was weighed into sterile Universal bottles, diluted with 2 ml sterile Maximum Recovery Diluent (MRD, Oxoid,

156 Basingstoke, UK), and mixed thoroughly. This constituted the 1:2 dilution (w/v). Further
157 serial dilutions were made in MRD and 10 µl of each dilution were inoculated on CCDA
158 and Brilliance CampyCount Agar plates (Oxoid, Basingstoke, UK), incubated
159 microaerophilically at 42°C for 24-48 hr and then assessed for the presence or absence of
160 thermotolerant *Campylobacter* species. The individual caeca from five birds per pen were
161 analysed in duplicate (i.e. two replicate samples analysed per bird). Plates of an
162 appropriate dilution were selected and putative colonies enumerated. As a confirmatory
163 measurement, two colonies from each presumptively positive plate were selected and sub-
164 cultured onto paired blood agar plates (Oxoid, Basingstoke, UK). These plates were
165 incubated at 37°C for 48 hr, one plate aerobically, one plate microaerophilically. The
166 presence of *Campylobacter* was indicated by a lack of growth aerobically and colonies
167 with *Campylobacter* morphology that grow microaerophilically. In addition, Gram stains
168 were carried out on all presumptively positive samples. As a further step, oxidase strips
169 (Oxoid, Basingstoke, UK) were used to confirm that samples were oxidase positive
170 (Cowan and Steel, 1965; Corry et al., 1995). The same series of samples were tested for
171 presence and absence of *E. coli* using chromogenic plates (Oxoid, Basingstoke, UK) and
172 incubated for 20 hr at 37°C, using the same procedure as reported for *Campylobacter*

enumeration. All results were expressed as colony forming units (CFU) per gram of caecal contents.

In addition, Polymerase chain reaction (PCR) was conducted on five representative colonies isolated from CCDA plates from each treatment group to confirm the presence of *C. jejuni* vs. *C. coli*. The primer sets in the multiplex PCR target the identification of *Campylobacter jejuni* and *Campylobacter coli* based on the amplification of the two genes, *mapA* (589 bp) *C. jejuni* and *ceuE C. coli* (462 bp). In addition, a 16S primer (800bp) set was included as quality assurance of the DNA-preparation and analysis (internal control). Between 3-4 colony morphotypes from each treatment group were examined. To avoid false negatives three different concentrations of each isolate's template were used for PCR amplification.

Minimum inhibitory concentration assays (Growth inhibition studies with *Campylobacter jejuni, Escherichia coli* and *Salmonella enterica*)

Ferric tyrosine was subjected to two digestive phases to mimic digestion in the broiler gut. Ferric tyrosine is poorly soluble and the digestive steps were included to enhance product solubility and bioavailability. The pepsin digestion phase was performed to mimic conditions in the acidic proventriculus and the pancreatin digestion phase to mimic

191 conditions in the neutral duodenum. In brief, 240 mg ferric tyrosine was suspended in 5
192 ml of 50 mM Na-phosphate buffer pH 6.5. Then 2.25 ml of 150 mM HCl and 0.75 ml of
193 activated pepsin (1 mg/ml) in 10 mM HCl were added; and the pH adjusted to pH 2.1.
194 The resulting suspension was digested for 1 hr at 37 °C. Following the pepsin digestion
195 phase, 4 ml of 150 mM $NaHCO_3$, 2 ml of bovine bile (125 mg/ml in 150 mM $NaHCO_3$)
196 and 2 ml of porcine pancreatin (12.5 mg/ml in 150 mM $NaHCO_3$) were added to the
197 digested suspension and the pH was adjusted to 6.5 with NaOH. The suspension was then
198 left to digest for 3 hr at 37 °C after which the total volume was adjusted to 20 ml. A
199 positive control (PC) was prepared by following the steps described above, with no added
200 ferric tyrosine. The two digests (PC digest and 20 mM ferric tyrosine digest) were
201 sterilized by UV light before use in the MIC studies.
202
203 For the MIC dilution study, *C. jejuni* strain DSM4688 grown in Müller-Hinton growth
204 medium, and *E. coli* strain 156/97 F4+ and *S. enterica* serovar Typhimurium strain IR715
205 both grown in Luria broth were added to 96-well microtitre plates (Merck, Germany)
206 containing the ferric tyrosine digest at concentrations ranging from 25.5 to 408 mg/L for
207 *C. jejuni* and 0.39 to 200 mg/L for *E. coli* and *S. enterica*, and the PC digest in dilutions
208 corresponding to the amounts of digest added with the ferric tyrosine. The range of

concentrations selected were chosen to meet or exceed the practical doses used in feed. All plates were incubated at 38ºC. Plates containing *C. jejuni* were read after 24 hr by measuring fluorescence with a Perkin Elmer multimode plate reader after rendering bacterial cells fluorescent with SYBR Green dye (Sigma Aldrich, Darmstadt, Germany). Plates containing *E. coli* and *S. enterica* were read at 4 and 20 hr. Turbidity was measured using a spectrophotometer at a wavelength 600 nm. The MIC value was defined as the lowest product concentration that yields >50% reduction in growth obtained in cultures with no added test product.

Statistical analyses

The pen was considered the experimental unit for zootechnical and microbiological data. The arithmetic means of body weight, average daily gain, average feed intake and feed conversion rate were calculated per pen. The bacterial counts were transformed to $\log_{10}$ prior to analysis. Zootechnical and microbiological data were analysed by one-way analysis of variance (ANOVA) using the General Linear Model (GLM) procedure in Unistat (Unistat Ltd., Version 6.5) according to the following model: $Yi = \mu + \alpha i + \varepsilon i$, where Yi was the dependent variable, μ was the overall mean, αi was the effect of treatment, and εi was the residual error. For zootechnical and microbiological data,

227 significant differences were declared at P≤0.05, while near significant trends were
228 considered for 0.05<P≤0.10. Arithmetic means were separated by Tukey's *post-hoc*
229 comparison test. Results are reported as arithmetic means, the treatment probability (P)
230 and the pooled standard error of the mean (SEM). If *Campylobacter* counts are randomly
231 distributed among individual birds and pens, the counts obtained should follow a Poisson
232 distribution, where variance equals the mean. If variance exceeds the mean this indicates
233 overdispersion and demonstrates that the counts are not homogenous. The distribution of
234 caecal *Campylobacter* spp. and *E. coli* counts were assessed for overdispersion by
235 multiplying the variance to mean ratio by the number of degrees of freedom, and
236 comparing the results with the chi-square distribution (Bliss and Fisher, 1953).
237 Overdispersion was confirmed when P<0.05).
238
239 **Results**
240 The effect of ferric tyrosine on broiler zootechnical performance during each study period
241 is summarised in Table 4. The mortality rate (including culled birds) was low and there
242 were no significant differences in mortality between treatment groups (T1, 6/210 (2.9%);
243 T2, 4/210 (1.9%); T3, 4/210 (1.9%); T4, 6/210 (2.9%)). The majority (13/20) of birds
244 were culled early in the study as poor or non-starters/small birds. During the first study
245 period (0 to 21 days on trial), broilers fed diets supplemented with ferric tyrosine (T2, T3

246 and T4) weighed significantly more at day 21 (+110 g, +130 g, +63 g; 630, 650, 583 *vs.*
247 520 g; P<0.001, P<0.001, P=0.002, respectively), and gained significantly more weight
248 (+110 g. +130 g, +63 g; 588, 608, 541 *vs.* 478 g; P<0.001, P<0.001, P=0.003,
249 respectively) compared to broilers fed the T1 Control diet. No significant differences were
250 noted in feed efficiency (Table 4). Similarly, during the second study period (22 to 42
251 days on trial), broilers fed T2, T3 and T4 diets weighed significantly more at study end
252 (+213 g, +190 g, +180 g; 2,081, 2,052, 2,048 *vs*. 1,868 g; P=0.002, P=0.008, P=0.009;
253 respectively) and broilers fed T2 and T3 diets consumed significantly more feed (+263 g,
254 +219 g; 2,751, 2,707 *vs.* 2,488 g; P=0.005, P=0.021; respectively) compared to broilers
255 fed the T1 Control diet. Broilers fed the T3 diet presented a significantly higher feed
256 conversion ratio (1.934 *vs.* 1.845, 1.827 g; P=0.014, P=0.003; respectively) compared to
257 broilers fed the T1 Control diet and the T4 diet. In addition, broilers receiving the T4 diet
258 tended to gain more weight (+117 g; 1,465 *vs.* 1,348 g; P=0.062; respectively) and to eat
259 more (+187 g; 2,675 *vs.* 2,488 g; P=0.057; respectively) compared to broilers fed the T1
260 Control diet. During the overall study period (0 to 42 days on trial) broilers fed the diets
261 containing ferric tyrosine (T2, T3 and T4) gained significantly more weight (+212 g, +182
262 g, +179 g; 2,039, 2,009, 2,006 *vs.* 1,827 g; P=0.002, P=0.008, P=0.009; respectively) and
263 ate significantly more feed (+385 g, +385 g, +258 g; 3,609, 3,609, 3,482 *vs.* 3,224 g;

264 P<0.001, P<0.001, P=0.027; respectively) compared to broilers fed the T1 Control diet.
265 No significant differences in feed efficiency were noted between the groups
266 supplemented with ferric tyrosine and the T1 Control group.
267 Microbiological counts from the caecal samples collected on day 42 are summarised in
268 Table 5. The results showed a significant reduction in *Campylobacter* spp. in birds fed
269 T3 and T4 diets compared to the birds fed the T1 Control diet (1.8 $\log_{10}$ reduction,
270 P<0.001 and 2.5 $\log_{10}$ reduction, P<0.001, respectively, Table 5 and Figure 1a) when
271 samples were grown on CCDA medium. Moreover, when samples were grown on
272 Brilliance medium, *Campylobacter* spp. counts were significantly reduced in birds fed
273 T2, T3 and T4 diets compared to the birds that were fed the T1 Control diet (1.2 $\log_{10}$
274 reduction, P=0.043; 2.4 $\log_{10}$ reduction, P=0.001 and 3.1 $\log_{10}$ reduction, P<0.001,
275 respectively, Table 5 and Figure 1b). There was a near-significant trend towards reduced
276 *E. coli* counts in broilers fed the T4 diet compared to broilers fed the T1 Control diet (1.3
277 $\log_{10}$ reduction, P=0.083, respectively, Table 5 and Figure 1c). All individual birds in T1
278 tested positive for *Campylobacter* spp. and *E. coli*. Furthermore, Figure 1 shows the
279 distribution of the counts for each treatment groups and demonstrates that all pens in T1
280 were positive for *Campylobacter* spp. and *E. coli*. Additionally, all birds from T2 and T3
281 had positive *Campylobacter* counts and only two birds from T4, each from different pens

282 (pen 4 and pen 13), had a negative *Campylobacter* count. However, the other birds tested
283 from pen 4 & 13 were positive. Analysis of the distribution of the counts in T1 birds and
284 T1 pens showed that the distribution conformed to a Poisson distribution, where the mean
285 and variance are equal, indicating that the counts were homogenous among control birds
286 and pens and there was no significant overdispersion of counts. In comparison, significant
287 overdispersion was observed for the *Campylobacter* counts from Brilliance media for T3
288 and T4 ($P=0.03$ and $P<0.001$, respectively).
289
290 Results from the PCR confirmed the presence of *C. jejuni* and *C. coli*.
291
292 The MIC value for *C. jejuni* was >400 mg/L (Table 6) and >200 mg/L for *E. coli* and *S.*
293 *enterica* (Table 7). After 24 hr incubation, *C. jejuni* fluorescence increased by 29% when
294 exposed to the PC digest at a dilution corresponding to 408 mg/L ferric tyrosine and
295 increased by 13% when exposed to ferric tyrosine digest at 408 mg/L (Table 6). After 20
296 hr incubation, the turbidity of *E. coli* decreased by 61% with PC digest dilution
297 corresponding to digest provided with 49.9 mg/L ferric tyrosine digest and decreased by
298 14% at 200 mg/L ferric tyrosine digest (Table 7). Similarly, *S. enterica* turbidity
299 decreased by 37% after 20 hr incubation when exposed to the PC digest at 200 mg/L, and

300 turbidity increased by 5% after 20 hr when exposed to 200 mg/L ferric tyrosine (Table
301 7).

302 **Discussion**

303 Here, the effects of ferric tyrosine on broiler zootechnical performance and caecal
304 *Campylobacter* spp. and *E. coli* were evaluated, along with an investigation into the MIC
305 of ferric tyrosine against *C. jejuni*, *E. coli* and *S. enterica*. The results from the present
306 study show that ferric tyrosine when administered in the feed of broilers, significantly
307 reduced caecal *Campylobacter* spp. (T3 and T4), reduced *E. coli* counts (T4), and
308 significantly improved weight gain at day 42, but did not affect FCR. Under the
309 conditions of this study, ferric tyrosine added to diets at 0.02 g/kg, 0.05 g/kg and 0.20
310 g/kg led to a 1.2 $\log_{10}$, 2.4 $\log_{10}$ and 3.1 $\log_{10}$ CFU/g reduction in caecal *Campylobacter*
311 spp. counts, respectively, when samples were grown on Brilliance media. These results
312 agree with those from a recent study that evaluated ferric tyrosine in broiler diets (Khattak
313 et al., 2018). In that study, the authors reported caecal *Campylobacter* reductions of 0.8
314 $\log_{10}$, 1.9 $\log_{10}$ and 2.0 $\log_{10}$ CFU/g in birds fed ferric tyrosine at 0.02 g/kg, 0.05 g/kg and
315 0.20 g/kg, respectively. A recent quantitative microbial risk assessment (QMRA)
316 estimated that reducing caecal colonisation of birds at flock level by 2 $\log_{10}$ or 3 $\log_{10}$
317 CFU/g could reduce the incidence of human campylobacteriosis attributed to

318 contaminated broiler meat by 76% and 90%, respectively (Romero-Barrios et al., 2013).
319 Another earlier QMRA estimated that the incidence of disease in humans could be
320 reduced by 48%, 85% and 96% if carcass contamination with *Campylobacter* can be
321 reduced by 1, 2 or 3 $\log_{10}$ CFU/g, respectively (Messens et al., 2007). According to these
322 figures, ferric tyrosine added to diets at 0.02 g/kg, 0.05 g/kg and 0.20 g/kg meets the
323 thresholds outlined in the two QMRAs, indicating that this product could be useful for
324 reducing the burden of *Campylobacter* on poultry farms, which may lead to a reduction
325 in broiler meat contamination at slaughter. Slight differences were observed in counts
326 when *Campylobacter* was grown on CCDA (*Campylobacter* Blood Free Selective Agar)
327 media, which can be used for the isolation of *Campylobacter jejuni*, *Campylobacter coli*
328 and *Campylobacter lari*. Brilliance CampyCount Agar is a medium specifically designed
329 for accurate, specific and easy enumeration of *Campylobacter jejuni and Campylobacter*
330 *coli* from poultry. It is a transparent medium on which *Campylobacter* produce distinct
331 dark red colonies, making identification and counting significantly easier than on
332 traditional charcoal or blood containing agar. PCR analysis confirmed the presence of *C.*
333 *jejuni* and *C. coli*. In addition to the reduction of caecal *Campylobacter*, a reduction in
334 caecal *E. coli* was also noted. Caecal *E. coli* counts were reduced by 1.0 $\log_{10}$, 0.7 $\log_{10}$
335 and 1.3 $\log_{10}$ CFU/g in birds fed ferric tyrosine at 0.02 g/kg, 0.05 g/kg and 0.20 g/kg feed,

336 respectively. These results agree with those of Khattak et al. (2018) who reported
337 reductions of 0.6 $\log_{10}$, 0.8 $\log_{10}$ and 1.2 $\log_{10}$ CFU/g, respectively. It has been suggested
338 that *E. coli* infection is established more easily in birds infected with *Campylobacter* (Bull
339 et al., 2008) and an epidemiological study reported increased *E. coli* in chicken carcasses
340 infected with *Campylobacter* (Duffy et al., 2014). Moreover, translocation of *E. coli* to
341 the liver, spleen and caecum increases in birds infected with *C. jejuni* (Awad et al., 2016).
342 This evidence would suggest that *Campylobacter* infection may positively influence the
343 establishment of other pathogenic microbial populations, which could have serious
344 implications for public health. In addition, the emergence of antibiotic resistance to
345 *Campylobacter* spp. in humans and animals underlines the need for non-antibiotic
346 alternatives to aid *Campylobacter* control on farms.
347
348 In this study, a natural challenge model was used, whereby study birds were housed in a
349 barn that had housed broilers that previously tested positive for *Campylobacter* spp. on
350 several occasions, and were placed in in pens containing dirty litter from an earlier study,
351 in which birds had tested positive for campylobacters. This study design did not quantify
352 the level of infection before or during the study. However, on day 42, all caecal samples
353 collected from control birds tested positive for *Campylobacter* spp., and the counts

354 followed a Poisson distribution indicating that the infection was homogenous among
355 individual birds and pens. Furthermore, as the layout of pens followed a randomised block
356 design, it is assumed that all pens were exposed to a similar level of *Campylobacter* spp.
357 challenge. It has been shown that a single bird harbouring low numbers of *Campylobacter*
358 can infect a whole flock, (Stern et al., 2001) and that once a flock becomes *Campylobacter*
359 positive, the surrounding environment becomes widely contaminated (Herman et al.,
360 2003) and contamination can persist for several weeks (Johnsen et al., 2006).
361
362 The MIC results presented in this study show that ferric tyrosine does not exert
363 antimicrobial activity against the strains of *C. jejuni*, *E. coli* and *S. enterica* tested. MICs
364 of >400 mg/L and >200 mg/L were reported for *C. jejuni* and *E. coli* and *S. enterica*,
365 respectively, which are much higher than MIC thresholds used to monitor antimicrobial
366 susceptibility and resistance. Furthermore, effective antimicrobials inhibit or kill
367 *Campylobacter* spp. at low concentrations. According to recent guidelines, cut-off values
368 for erythromycin, tetracycline and ciprofloxacin against *Campylobacter jejuni* are ≤4
369 mg/L, ≤2 mg/L and ≤0.5 mg/L, respectively, while the cut-off values for ampicillin,
370 ciprofloxacin and colistin when tested against *Salmonella* spp. and *E. coli* are ≤8 mg/L,
371 ≤0.06 mg/L and ≤2 mg/L, respectively (ECDC, 2016). This study has shown that ferric

372 tyrosine does not inhibit or kill *Campylobacter* spp. at concentrations up to 400 mg/mL,
373 which is much higher than the ferric tyrosine concentration in the feed or broiler gut.
374 Hence, these results indicate that ferric tyrosine does not exhibit classic antibiotic activity
375 at up to 400 mg/mL.
376
377 Significant improvements in final body weight and weight gain were observed in the birds
378 fed ferric tyrosine in comparison to the birds fed the control diet. Similar results were
379 observed in the study conducted by Khattak et al. (2018). *C. jejuni* infection can
380 significantly impair the growth performance of poultry (Awad et al., 2014a,) and a highly
381 significant negative association between *Campylobacter* and feed efficiency has been
382 reported (Sparks, 2016). *Campylobacter* infection downregulates the gene expression of
383 various carrier proteins responsible for the absorption of nutrients (Awad et al., 2014b),
384 leading to decreased nutrient adsorption and reduced growth performance.
385
386 Aspects of *Campylobacter* pathogenesis remain poorly understood, particularly
387 molecular host-pathogen interactions. Human histo-blood group antigens (BgAgs) are
388 often targeted by mucosal organisms to aid adherence prior to invasion. The BgAgs-
389 binding adhesins of *C. jejuni* have been identified as the major subunit protein of the

390 flagella (FlaA) and the major outer membrane protein (MOMP) (Mahdavi et al., 2014).
391 MOMP is a member of the trimeric bacterial porin family that assists the mucosal
392 adhesion and invasion of *C. jejuni* (Mahdavi et al., 2014). Porins are involved in the
393 uptake of nutrients through the outer membrane by passive diffusion along concentration
394 gradients (Ferarra et al., 2016). MOMP is also able to bind to multiple host cell
395 membranes by promoting biofilm formation and auto-aggregation. The actual mode of
396 action of ferric tyrosine is unknown, but some bacteria use specific outer membrane
397 receptors to uptake ferric iron. It is thought that ferric tyrosine may be able to bind to
398 MOMP and block the interaction of MOMP on the surface of *Campylobacter* with the
399 BgAgs of the gastrointestinal epithelial cells. As a result, it prevents *Campylobacter*
400 colonization of the avian gut by reducing biofilm formation. A recent study has
401 demonstrated that ferric tyrosine inhibits biofilm formation *in vitro* (Khattak et al., 2018),
402 which supports the assumed mode of action.
403
404 *Campylobacter* remains a real threat to public health. With prophylactic administration
405 of antibiotics at farm level no longer a viable control option due to increasing antibiotic
406 resistance, there is a critical need to find non-antibiotic alternatives that can be used in
407 conjunction with on-farm biosecurity measures to reduce *Campylobacter* colonisation of

poultry flocks. In conclusion, the results from the present study illustrate that ferric tyrosine can significantly reduce caecal *Campylobacter* spp. and *E. coli* and improve bird weight gain, indicating that this feed additive may contribute to control of *Campylobacter* spp. under commercial poultry production conditions.

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535 **Table 1.** Experimental diets

Treatment	Ferric tyrosine, g/kg feed	Microtraced[1] Ferric tyrosine g/kg feed
T1	Control – 0 g/kg	0
T2	T1 + 0.02 g/kg feed	0.022[2]
T3	T1 + 0.50 g/kg feed	0.055[2]
T4	T1 + 0.20 g/g/feed	0.220[2]

[1]1 g of microtracer contains 60,000 violet graphite particles
[2]Microtracers at 10% in test products

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538 **Table 2.** Feed composition and calculated analyses

Ingredients (%)	Starter Mash 1-21 days of age	Grower Mash 22-42 days of age
Wheat	69.862	67.354
Barley	-	7.5
Soybean meal, 48% CP	23.4	21.4
Sodium bicarbonate	0.13	0.22
Fishmeal 66%	2.5	-
Soy oil	1.3	1.4
L-lysine HCl	0.128	0.175
DL-methionine	0.123	0.164
Choline chloride	0.067	0.067
Dicalcium phosphate	0.13	0.32
Calcium carbonate	1.74	0.74
Sodium chloride	0.12	0.16
Minerals and vitamins[1]	0.5	0.5
Total	100	100
Calculated analyses (%, unless specified differently)		
ME Broiler, MJ/kg	11.526	12.346
Crude protein	21	19
Crude fibre	2.73	2.914
Ash	5.796	4.571
Dry matter	72.97	77.503
Crude fat	3.0	3.0
Lysine	1.18	1.050
Methionine	0.45	0.438
Methionine + cysteine	0.797	0.766
Threonine	0.75	0.661
Tryptophan	0.259	0.237
Calcium	1.102	0.651
Sodium	0.126	0.142

[1]Supplies per kg feed: Vit A: 0.010 MIU; Vit D_3: 0.005MIU; Vit E: 50mg; Vit K_3: 3 mg; Vit B_1: 2.0 mg; Vit B_2: 7 mg; Vit B_6: 5 mg; Vit B_{12}: 15 µg; Folic acid: 1.0 mg; Biotin: 0.2 mg; Pantothenic acid: 15 mg; 3a315 niacinamide: 50 mg; Mo 0.5 mg Mn: 100 mg; Zn: 80 mg; I: 1.0 mg; Cu: 10 mg; Se: 0.20 mg, Fe: 267 mg

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541 **Table 3**. Analysed values of experimental diets

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Sample	Diet	Ferric tyrosine, g/kg	Moisture (%)	Crude protein (%)	Ether extract (%)	Ash (%)	Fe (mg/kg)	Ferric tyrosine-microtracer (% recovery[1])	Calculated ferric tyrosine content (g/kg)
T1	Starter	0	11.6	20.4	3.1	5.5	125	NA	NA
T2		0.02	11.5	20.3	2.9	5.4	189	131	0.026
T3		0.05	11.3	20.6	2.9	5.5	198	118	0.059
T4		0.20	11.3	20.5	2.9	5.3	196	107	0.214
T1	Grower	0	11.9	19.1	3.2	4.2	171	NA	NA
T2		0.02	11.9	18.7	3.0	4.2	159	98	0.020
T3		0.05	11.9	18.7	3.0	4.7	185	85	0.043
T4		0.20	11.5	19.1	3.0	4.4	166	81	0.162

543 NA – Not applicable; [1]Calculated ferric tyrosine = % recovery of microtracer x ferric tyrosine dose

Table 4. Effect of dietary addition of ferric tyrosine on broiler zootechnical performance parameters for each study period.

Parameter	Treatment				SE	Treatment P-value (ANOVA)
	T1 0 g/kg ferric tyrosine	T2 0.02 g/kg ferric tyrosine	T3 0.05 g/kg ferric tyrosine	T4 0.20 g/kg ferric tyrosine		
BW 1 d (g)	41.81	42.00	42.48	42.19	0.135	0.364^{NS}
BW 21 d, (g)	520^{a}	**630^{c}**	**650^{c}**	**583^{b}**	11.6	**0.001**
BW 42 d (g)	1,868^{a}	**2,081^{b}**	**2,052^{b}**	**2,048^{b}**	24.0	**0.001**
AWG 1-21 d (g)	478^{a}	**588^{c}**	**608^{c}**	**541^{b}**	11.5	**<0.001**
AWG 22-42 (g)	1,348^{x}	1,451^{xy}	1,401^{xy}	*1,465^{y}*	17.2	*0.055*
AWG 1-42 d (g)	1,827^{a}	**2,039^{b}**	**2,009^{b}**	**2,006^{b}**	24.0	**0.002**
AFI 1-21 d (g)	737^{a}	**859^{bc}**	**901^{c}**	**807^{ab}**	16.5	**<0.001**
AFI 22-42 (g)	2,488^{a,x}	**2,751^{b}**	**2,707^{b}**	2,675^{ab,y}	30.8	**0.005**
AFI 1-42 d (g)	3,224^{a}	**3,609^{b}**	**3,609^{b}**	**3,482^{b}**	42.8	**<0.001**
FCR 1-21 d (g)	1.539	1.462	1.484	1.488	0.0138	0.240^{NS}
FCR 22-42 (g)	**1.845^{a}**	1.898^{ab,y}	**1.934^{b}**	**1.827^{a,x}**	0.0124	**0.002**
FCR 1-42 d (g)	1.765^{xy}	1.771^{xy}	*1.798^{y}*	*1.736^{x}*	0.0087	*0.083*

Results show least square mean of 6 replicate pens. Nº replicates/treatment = 6 pens of 35 male birds/ treatment; Means separated by Tukey Test. SE = Standard error; BW = mean bird body weight; AWG = mean pen weight gain; AFI = mean pen feed intake; FCR = feed/gain; NS – not significant. Values in same column with no common abc superscript are significantly different (P≤0.05); Values in same column with no common xy superscript exhibit a near-significant trend (0.05<P≤0.10). Text in bold = significant result (P≤0.05); text in italics = near-significant trend (0.05<P≤0.10).

552 **Table 5.** Caecal *Campylobacter* spp. and *E. coli* counts at 42 days of age (log_{10} CFU/g)

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Treatment	Dose g/kg	*Campylobacter* spp.		*E. coli*
		Caeca[1]	Caeca[2]	Caeca
T1 Control	0	5.879^{c}	4.799^{c}	6.438^{y}
T2 Ferric tyrosine	0.02	4.989^{bc}	3.621^{b}	5.449^{xy}
T3 Ferric tyrosine	0.05	4.104^{ab}	2.399^{a}	5.736^{xy}
T4 Ferric tyrosine	0.20	3.366^{a}	1.681^{a}	5.118^{x}
SEM		0.1301	0.1448	0.1843
Treatment P-value (ANOVA)		**<0.001**	**<0.001**	0.104

Nº replicates = 6 replicate pens per treatment. Results show group least square mean of 6 replicate pens
[1] Caecal samples cultured on CCDA medium; [2] Caecal samples cultured on Brilliance medium, SEM = standard error of the mean.
Values in same column with no common abc superscript are significantly different ($P \leq 0.05$)
Values in same column with no common xy superscript exhibit a near-significant trend ($0.05 < P \leq 0.10$)

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Table 6. Effect of ferric tyrosine on the growth of *Campylobacter jejuni* DSM4688 and minimum inhibitory concentrations (MIC).

Bacterium	Ferric tyrosine (mg/L)	Fluorescence after 24h incubation ($x10^{-6}$)		MIC (mg/L)
		[1] Positive control digest	Ferric tyrosine digest	
C. jejuni	25.5	2.58	2.38	> 400
	51	2.90	3.75	
	102	2.82	2.83	
	204	3.27	3.41	
	408	3.34	2.68	

[1] No product was added to the positive control digest. The concentration shown indicates that dilution of the digest was the same as that used for the corresponding ferric tyrosine digest.

Table 7. Effect of ferric tyrosine on the growth of *Escherichia coli* 156/97 F4+ and *Salmonella enterica* serovar Typhimurium strain IR715 and the minimum inhibitory concentrations (MIC).

Bacterium	Ferric tyrosine (mg/L)	Turbidity at 600 nm				MIC (mg/L)
		[1]Positive control digest		Ferric tyrosine digest		
		4h growth	20h growth	4h growth	20h growth	
E. coli	0.39	0.20	0.38	0.19	0.36	> 200
	0.78	0.17	0.38	0.18	0.34	
	1.56	0.16	0.35	0.18	0.36	
	3.12	0.15	0.32	0.19	0.36	
	6.24	0.15	0.29	0.20	0.37	
	12.5	0.15	0.23	0.20	0.37	
	24.9	0.16	0.20	0.20	0.37	
	49.9	0.14	0.15	0.20	0.36	
	99.8	0.15	0.15	0.19	0.33	
	200	0.17	0.15	0.18	0.31	
S. enterica	0.39	0.25	0.65	0.28	0.61	> 200
	0.78	0.26	0.64	0.27	0.61	
	1.56	0.25	0.65	0.28	0.61	
	3.12	0.22	0.62	0.28	0.62	
	6.24	0.24	0.63	0.28	0.62	
	12.5	0.25	0.61	0.28	0.62	
	24.9	0.25	0.61	0.27	0.63	
	49.9	0.26	0.60	0.27	0.62	
	99.8	0.26	0.49	0.24	0.61	
	200	0.25	0.41	0.23	0.64	

[1] No product was added with the positive control digest. The concentration shown indicates that dilution of the digest was the same as that used for the corresponding ferric tyrosine digest.

571 **Figure captions**

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573 **Figure 1.** Boxplots showing the distribution of caecal *Campylobacter* spp. and *E. coli*
574 counts at Day 42: a. Caecal *Campylobacter* spp. counts (log_{10} CFU/g) grown on CCDA
575 media, **b.** Caecal *Campylobacter* spp. counts (log_{10} CFU/g) grown on Brilliance media,
576 **c.** Caecal *E. coli* counts (log_{10} CFU/g) grown on chromogenic media

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